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Melbourne, Australia

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14. ABSTRACT Up to 80% of ASD patients exhibit gastrointestinal (GI) problems, but the underlying mechanisms are unknown. Many ASD associated mutations modify synaptic proteins and hence alter synaptic function in the brain. We propose that some of these mutations also alter the enteric nervous system (ENS) to produce bowel disorders. NL3 mice express a neuroligin-3 mutation identified in ASD patients and are more responsive to the GABA neurotransmission in the brain. This work aims to examine the spatiotemporal distribution patterns of NL3 and related proteins and mRNA in gut tissue from these mice in order to determine biological mechanisms contributing to GI dysfunction in ASD patients. We show expression of synaptic genes CNTNAP2, gephyrin and SHANK3, in mouse enteric tissue and upregulation of genes implicated in ASD and epilepsy during development of the mouse ENS. Neuronal proportions labelled for GABA, nNOS are similar in WT and NL3 adult mouse colon suggesting that the NL3 mutation impacts on synaptic function in the ENS without altering neuronal numbers during development. An unexpected finding from this work is that NL3 is present in presynaptic terminals and should lead to re-evaluation of its location at central synapses.

15. SUBJECT TERMS					
genes implicated in ASD are upregulated during gut development presynaptic localization of the neuroligin-3 protein					

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Introduction

Disorders in bowel function are common in autism. Up to 80% of ASD patients exhibit gastrointestinal (GI) problems, notably chronic constipation, but the underlying mechanisms are unknown. Many mutations identified in ASD patients modify synaptic proteins and hence alter synaptic function in the brain. We propose that some of these mutations also alter the enteric nervous system (ENS) of the GI tract to produce bowel disorders. We have shown that adhesion molecules important to synaptic function in brain including neuroligin-3 and neurexins 1 and 2 are expressed in mouse myenteric plexus. NL3 mice, which express a human neuroligin-3 mutation identified in ASD patients, are more responsive to the inhibitory neurotransmitter GABA in the brain. Our initial studies found that blockers of $GABA_A$ receptors, bicuculline and gabazine, depress motor activity in NL3 mouse colon, but have a lesser effect in wild type colon. This project aims to further investigate changes in gastrointestinal function in NL3 mice compared with WT littermates by examining the spatiotemporal distribution patterns of NL3 and related proteins and mRNA in gut tissue from these mice. This project aims to determine biological mechanisms contributing to gastrointestinal dysfunction in patients with ASD.

Body

Identifying developmental expression of neuroligin 3

Task 4. Time course of expression of neuroligin 3. PI Young (RT-PCR, FACS, immunohistochemistry, confocal microscopy; 22
months: months 3-24)
4a. qRT-PCR experiments in developmental tissue. 30 NL3 mice (5 months: months 3-7).
4b. FACS experiments developmental tissue. 15 Ret-GFP mice (5 months: months 8-12)
4c. Immunohistochemistry in developmental tissue (12 months: months 13-24).

PCR experiments in developmental tissue

This work refers to **SOW task 4a. qRT-PCR experiments in developmental tissue.**

To characterise synaptic function in the enteric nervous system, it is essential to determine whether relevant synaptic genes expressed in the CNS are also expressed in the GI system of the mouse. In particular, it is unknown whether synaptic genes that serve as markers of synaptic function (eg gephyrin, a marker of GABAergic synapses in the CNS) and synaptic genes associated with ASD in patients (eg CNTNAP2 and SHANK3) are expressed in the ENS of NL3 mice at similar levels to those in WT mice. We have designed PCR primers to detect CNTNAP2 and SHANK3 and established that their mRNA is expressed in colon and duodenum from adult C57Bl/6 mice (**Figure 1**). We have also established, using published primer sequences for gephyrin mRNA (Gkogkas et al., 2012), that this inhibitory synaptic marker is expressed in colon and duodenum of adult C57Bl/6 mice (**Figure 1**).

Table 1: Primers used for PCR (see Figure 1).

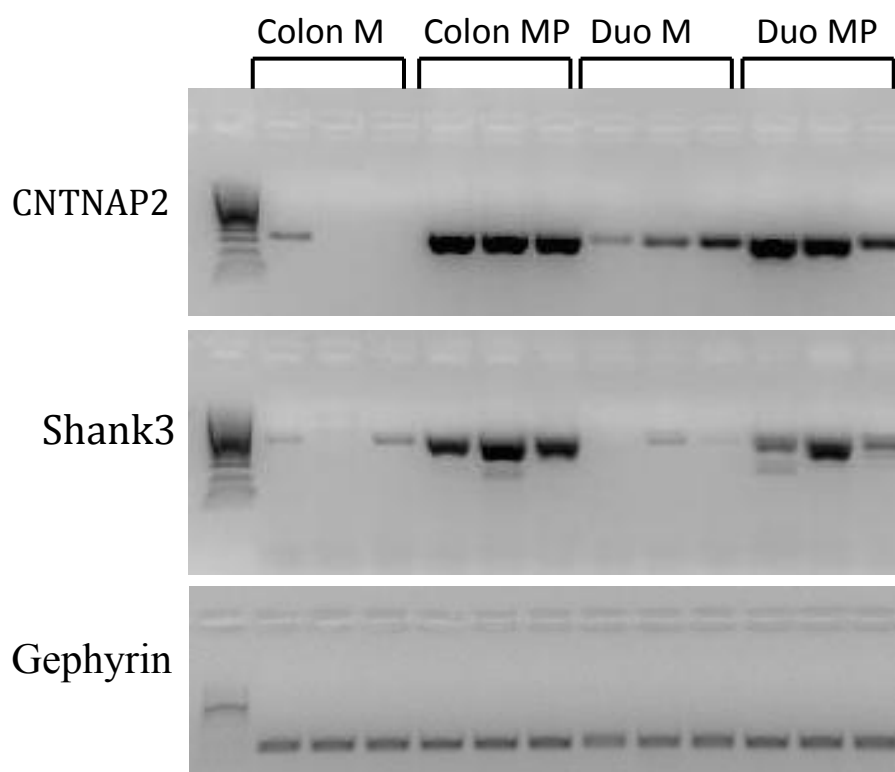| Length	Name	Sequence	Product
20bp	Cntnap2 fwd	gggtgagctggtggaatcta	
20bp	Cntnap2 rev	gtgaagttggggtcgttgtt	340bp
20 bp	Shank3 fwd	GGGCTCTCTGCGGAAGGGGA	
20 bp	Shank3 rev	GGCGCTGTGGTTTCGACGGA	537bp
24bp	Gephyrin fwd	ATGATCCTCACCAACCACGACCAT	
24bp	Gephyrin rev	TGCCGATATAGTCCCACCCAACAA	150bp

Figure 1: PCR for mRNA encoding synaptic proteins are present in mouse myenteric plexus with associated smooth muscle. The expression of CNTNAP2, SHANK3 and Gephyrin mRNA was detected in enteric tissue preparations from adult C57Bl/6 mice. Expression of CNTNAP2 and SHANK3 was predominantly expressed in the myenteric plexus with lower levels of expression in the mucosa in both the colon and duodenum. Gephyrin, a marker for GABAergic synapses is expressed in both mucosal and myenteric plexus samples in both regions of the gasterointestinal system. Colon M: colonic mucosa, Colon MP: colonic myenteric plexus, Duo M: duodenal mucosa, Duo MP: duodenal myenteric plexus.

In addition to investigating the expression of a subset of the synaptic genes associated with ASD (**Figure 1**), we have designed PCR primers for 2 other members of the Shank family of postsynaptic scaffolding proteins (Shank1 and Shank2; **Table 2**) each of which have been associated with ASD (reviewed by Guilmatre et al., 2013).

Table 2: PCR primers for relevant synaptic function genes. Primers targeting Shank1 and Shank2 have also been designed for use in PCR experiments.

qPCR in developing mouse gastrointestinal tissue:

We first ran qPCR on an array of genes encoding ion channels to assess expression and fold difference during development. Gene expression levels in enteric neural crest cells from E11.5 mice were compared to expression levels from samples derived from mice aged E14.5, P0 and adult. The expression of a number of genes coding for ion channels important in synaptic transmission and neuronal excitability including the Calcium channel Cacna1c, the potassium channel Kcnq3 and the sodium channel alpha subunit Scn1a were identified and shown to increase during development. Mutations in these genes have been associated with ASD and epilepsy in patients (Betancur, 2009; Sicca *et al.*, 2011, Lee et al., 2012, Laumonnier et al., 2006, Gilling et al., 2013), which suggests that there may be gastrointestinal phenotypes in patients with epilepsy that have autism.

Methods:

i) RNA extraction: Enteric neural crest cells were FACS sorted from E11.5 and E14.5 using *Ednrb-hKikGR* mice (Nishiyama et al., 2012). At E11.5 and E14.5, the entire gut was collected. FACS sorted cells were collected in phosphate buffered saline (PBS), pelleted, excess PBS removed and immediately frozen at -80C. To prepare duodenum myenteric preparations, the small intestine was isolated from P0 and adult (C57Bl6 mice) in sterile DMEM/F12 media, and the mucosa and submucosa removed with forceps. The remaining

muscle, myenteric plexus and serosa were immediately transferred into 1ml of RNAlater (Qiagen).

Total RNA was extracted from approximately 1×10^6 purified E11.5 and E14.5 enteric neural crest cells using Qiashredder and RNeasy mini kit (Qiagen), including the on-column DNase treatment, according to manufacturer's instructions. Total RNA was extracted from P0 and adult gut using Trizol (Life Technologies Invitrogen), then purified further using RNeasy mini columns and on-column DNase treatment (Qiagen), according to manufacturer's instructions. RNA quality and quantity were tested by spectrophotometry using a NanoDrop 1000 and electrophoresis, and only RNA meeting the criteria detailed by SABiosciences RT^2 Profiler PCR Array System was used in the arrays.

ii) PCR array: 0.2μg of total RNA was converted to cDNA for each age, using the RT^2 First Strand kit (SA Biosciences). Real time PCR was performed on a 384 well RT^2 Profiler PCR array for Ion channels and Transporters (PAMM-036, 2011, SA Biosciences) using SA Biosciences RT^2 qPCR Master Mix, and run on an ABI 7900HT Real time instrument. Three separate PCRs were performed, in which cDNA from each age was loaded onto 96 wells of the 384 well PCR plate.

Real time PCR was run and analysed according to SA Biosciences recommended protocols, and data analysed using the SA Biosciences web portal data analysis.

Results:

Table 3: Gene regulation in comparison to E11.5 tissue. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change and fold-regulation values greater than 2 are indicated in red; fold-change values less than 0.5 and fold-regulation values less than -2 are indicated in blue. Control group refers to tissue harvested from mice aged E11.5.

		Up-Down Regulation (comparing to E11.5)		
		E14.5	PND0	Adult
Well	Symbol	Fold Regulation	Fold Regulation	Fold Regulation
B02	Cacna1c	4.1281	46.7486	54.79
F06	Kcnq3	169.9809	295.9111	110.5013
F11	Scn1a	12.9529	7.4023	1.8585

Table 4: P-values in comparison to E11.5 tissue. The p values are calculated based on a Student's t-test of the replicate 2^(- Delta Ct) values for each gene in the control group and treatment groups, and p values less than 0.05 are indicated in red.

		p-value (comparing to E11.5)		
Well	Symbol	E14.5	PND0	Adult
B02	Cacna1c	0.00049	0.000096	0.000789
F06	Kcnq3	0.000194	0.000372	0.000077
F11	Scn1a	0.009429	0.023087	0.414567

Immunohistochemistry in developmental tissue

This work refers to **SOW task 4c. Immunohistochemistry in developmental tissue.**

To test whether the altered GABA receptor function seen in our pharmacology experiments might be due to changes in the numbers of GABA neurons, we undertook immunostaining for GABA and the pan neuronal marker, Hu (**Figures 2 and 3**). These data showed no differences in the numbers of neurons (**Figure 4, Table 5,**) or in the proportions of neurons immunoreactive for GABA**,** neuronal nitric oxide synthase; nNOS (a marker of inhibitory motor neurons and some interneurons) or both between NL3^{R451C} and WT across the proximal, mid and distal colon (**Figures 2, 3 and 4**). These findings have important developmental implications for the NL3 mutation in the ENS as previous studies have shown clear changes in neuronal number and in the proportion of GABA neurons as a result of other genetic mutations influencing synaptic function during enteric development (Li et al., 2012). In effect, we can conclude that the NL3^{R451C} mutation does not affect differentiation of neural crest cells into enteric neurons and is unlikely to produce gross changes in their neurochemistry.
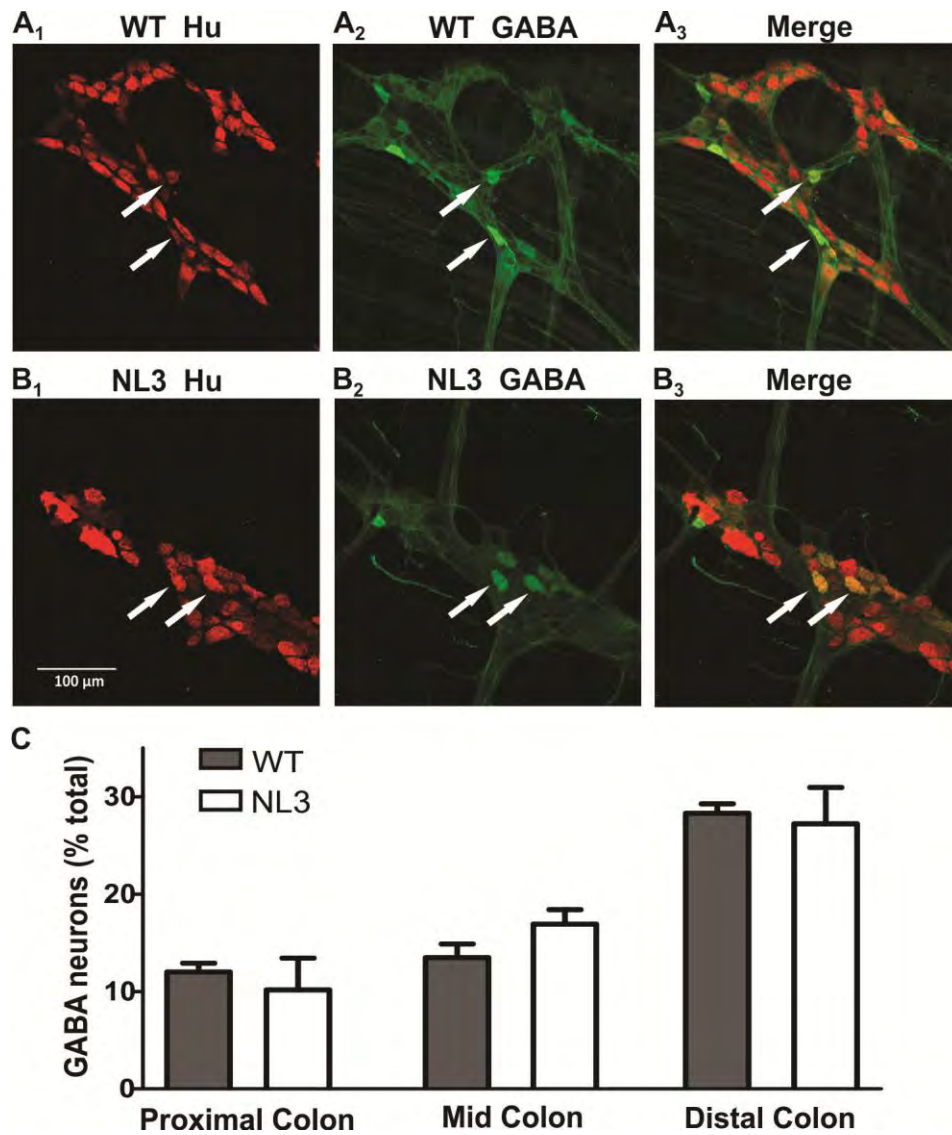
Figure 2: GABA labelling in the myenteric plexus of WT and NL3 mice colon.

We observed no change in neuronal proportions immunoreactive for GABA in NL3^{R451C} (n = 3) compared to WT (n = 3) colon. Representative images of myenteric plexus of (**A**) WT and (**B**) NL3^{R451C} distal colon illustrating neurons immunoreactive for Hu (A_1, B_1; red) and GABA (A_2, B_2; green) and merged images (A_3, B_3). Arrows indicate neurons labelled for Hu and GABA. **C**: Bar graph indicates the percentage of Hu-positive neurons immunoreactive for GABA in WT and NL3^{R451C} proximal, mid and distal colonic regions. There is an effect of region ($p<0.0001$). Data shown as mean ± s.e.m.
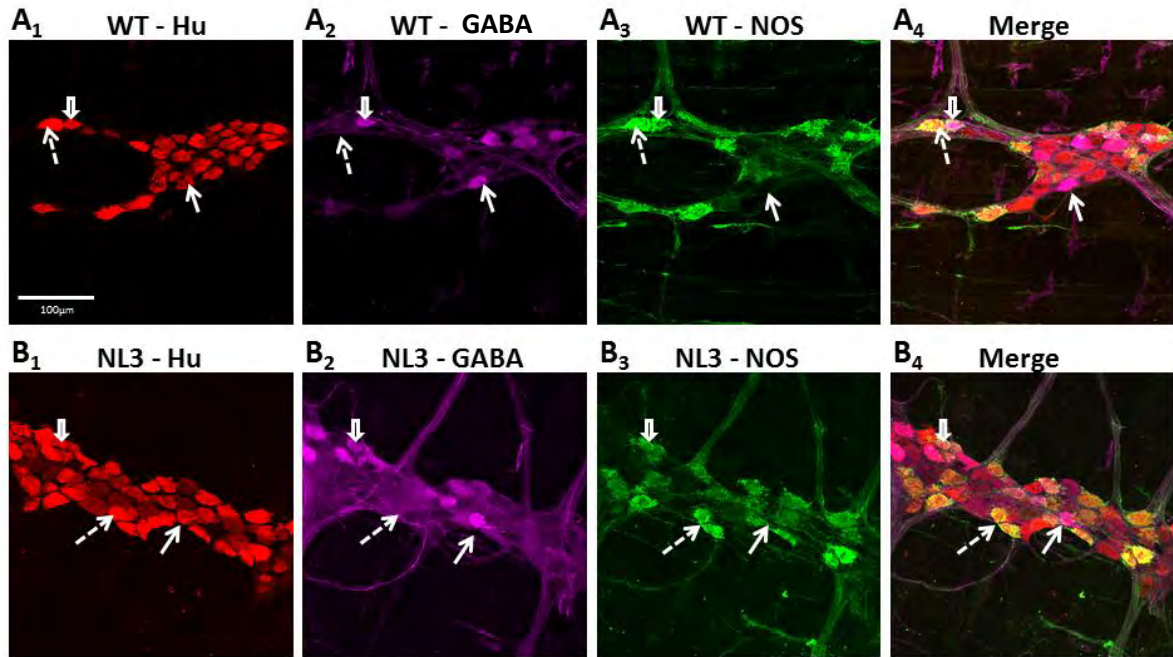
Figure 3: GABA, NOS and Hu labelling in WT and NL3 mouse distal colon.
Representative images of immunohistochemical labelling for the pan-neuronal marker
Hu, GABA, and nNOS in myenteric plexus from mouse distal colon of WT mouse.
Immunohistochemical staining for **A.** Hu (pan neuronal marker). **B.** GABA. **C.** nNOS.
D. Merged image demonstrating Hu, GABA and NOS immunoreactivity. Thin arrows:
Hu and GABA immunoreactive neurons. Dashed arrows: Hu + NOS immunoreactive
neurons. Thick arrows: Hu + GABA + NOS immunoreactive neurons. NL3: NL3[R451C]
KI mouse. Scale bar = 100 μm.

Table 5: Cell count data from proximal, mid and distal colon tissue preparations from WT (n=3) and NL3 (n=3) adult mice. Neurons were identified by their immunoreactivity for Hu, a pan neuronal marker. Cell populations that labelled for Hu in addition to GABA, nNOS or GABA together with nNOS were quantified for each region of the colon.

Proximal Colon

Animal	WT				NL3			
	HU	GABA only	NOS only	NOS + GABA	HU	GABA only	NOS only	NOS + GABA
1	428	33	101	22	333	16	97	4
2	306	16	90	15	330	16	109	10
3	284	31	98	6	349	33	127	25

Mid Colon

Animal	WT				NL3			
	HU	GABA only	NOS only	NOS + GABA	HU	GABA only	NOS only	NOS + GABA
1	632	60	244	41	607	22	229	65
2	754	53	269	44	892	107	285	41
3	941	48	175	58	767	71	175	79

Distal Colon

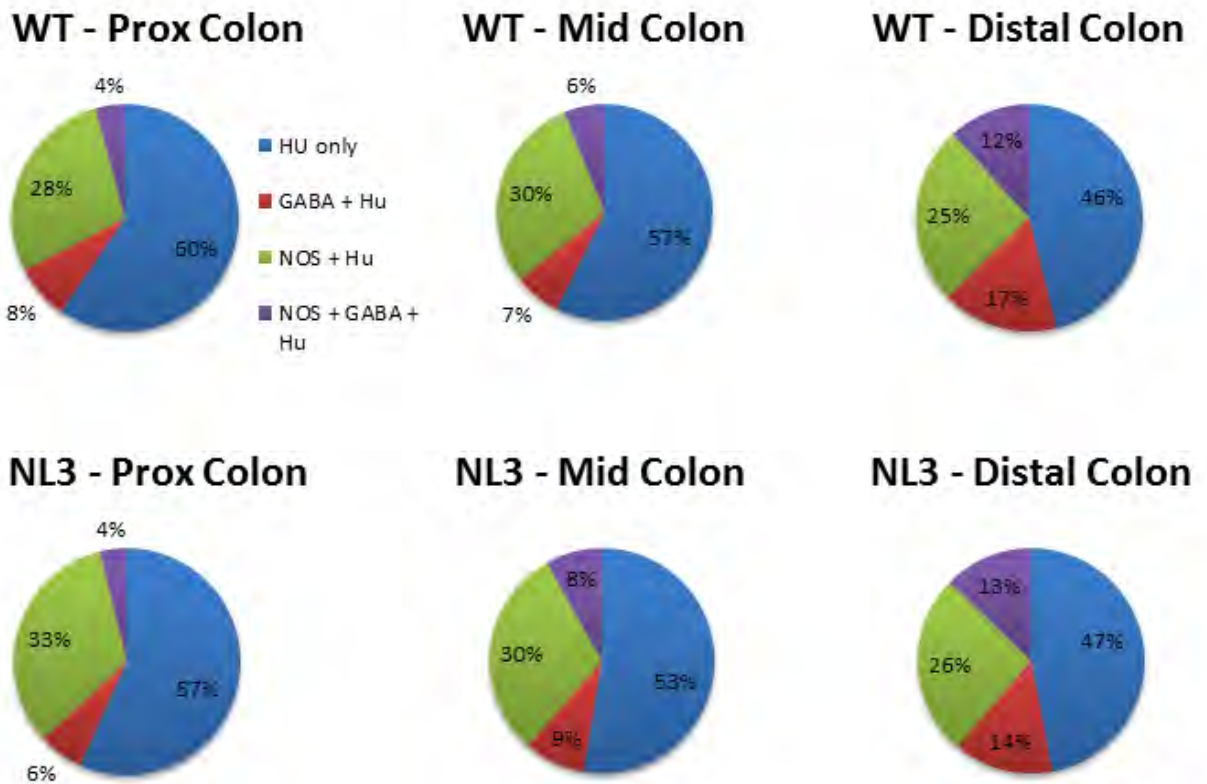| Animal	WT				NL3			
	HU	GABA only	NOS only	NOS + GABA	HU	GABA only	NOS only	NOS + GABA
1	517	98	132	55	477	55	120	57
2	491	69	131	68	388	56	116	39
3	436	73	105	47	424	74	98	69

Figure 4: Summary of cell count data from adult mouse colon tissue labelled for Hu, GABA and NOS. Data for neuronal populations labelling for GABA and NOS are represented as a proportion of total neurons (as identified as labelling positive for the pan neuronal marker, Hu). Raw cell count data is shown in Table 5. Prox = proximal.

We have performed fluorescence immunohistochemistry for neuroligin-3 in mouse myenteric plexus (**Figure 5**). Unexpectedly, these data show that neuroligin-3 is expressed in presynaptic terminals (see arrows, **Figure 5**, top panel). In addition, some neurons that were labelled for NOS were immunonegative for NL3 indicating that a subset of neuronal cell bodies expresses the NL3 protein in these preparations. Due to these surprising results, we then tested for NL3 staining in guinea pig colon myenteric plexus (using funds allocated for other projects) for comparison (**Figure 6**).

Immunolabeling for NL3 in guinea pig showed clear staining in neuronal cell bodies as well as in some processes within the myenteric plexus. Similar to reports in cultured hippocampal cells suggesting that NL3 is ubiquitously expressed in neurons (Budreck and Sheiffele 2007), our data in the guinea pig myenteric plexus show that NL3 staining is present in both nNOS immunoreactive cells as well as neurons that are not labelled for nNOS (see horizontal arrow

in **Figure 6** identifying a large neuronal cell body labelled for NL3 that is immunonegative for nNOS).

In addition to optimizing staining protocols for the NL3 antiserum, we have purchased antisera targeting Neurexin 1, Neurexin 2 and Neuroligin1 and have begun optimizing them for use in mouse enteric tissue.

Because NL3 was not present where expected (i.e. NL3 was expressed in presynaptic varicosities rather than on dendritic membranes), we have verified the specificity of the commercial antibody by Western blot (**Figure 7**), which has delayed the immunohistochemistry part of the project (i.e. in adult tissue). Specifically, characterising the cell classes expressing NL3 in Task 2 and NL3 expressing synapses in Task 2C have been delayed.

As a result, the developmental immunohistochemical study has been delayed because we needed to first verify the specificity of the antiserum and then confirm the presynaptic localization in adult tissue. The second component (synaptic labelling in Task 2C) is yet to be completed.
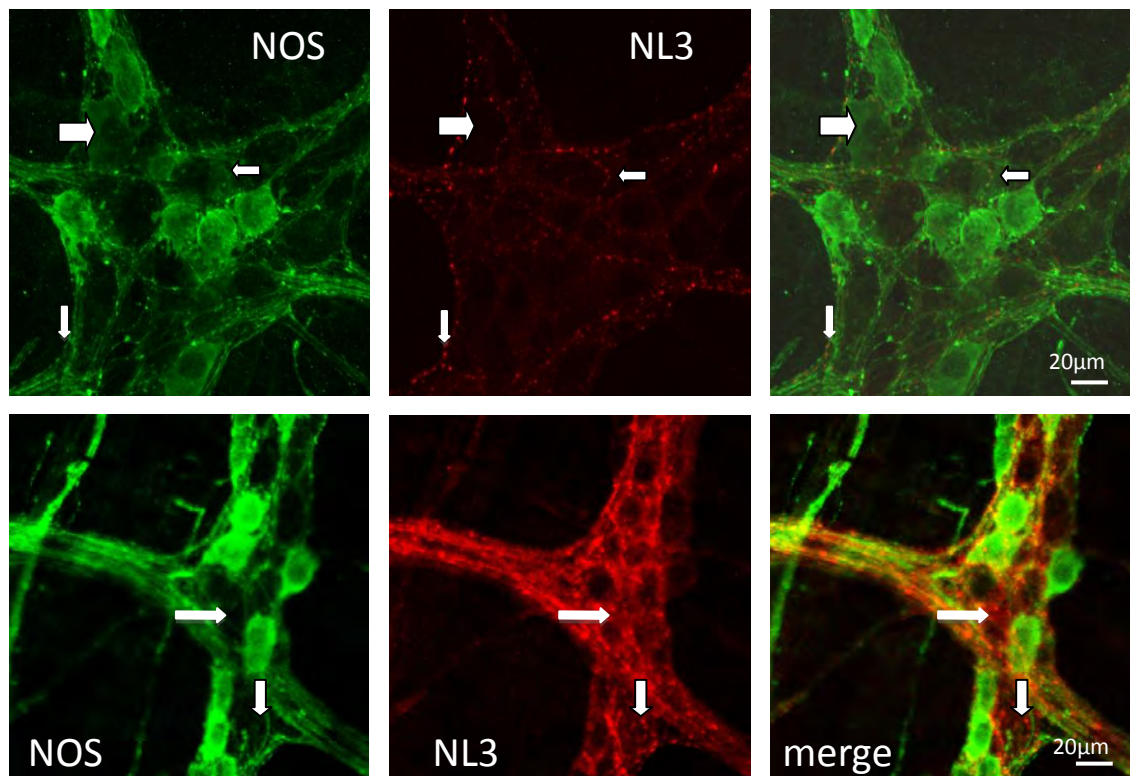
Figure 5: Localisation of nNOS and NL3 in the myenteric plexus of WT mouse colon.
Representative images of immunohistochemical staining for nNOS and NL3 in WT adult mouse myenteric plexus. **Top;** thick arrow identifies a NOS positive neuron which is negative for NL3, thin arrows identify beaded processes (putative axonal synaptic specializations) immunoreactive for both NOS and NL3. **Bottom;** horizontal arrow denotes a NOS negative neuron which is clearly labelled for NL3. Vertical arrow: NL3 immunoreactive (NOS negative) presynaptic specializations. Images acquired at 40x magnification (top panel), 63x magnification (bottom panel).
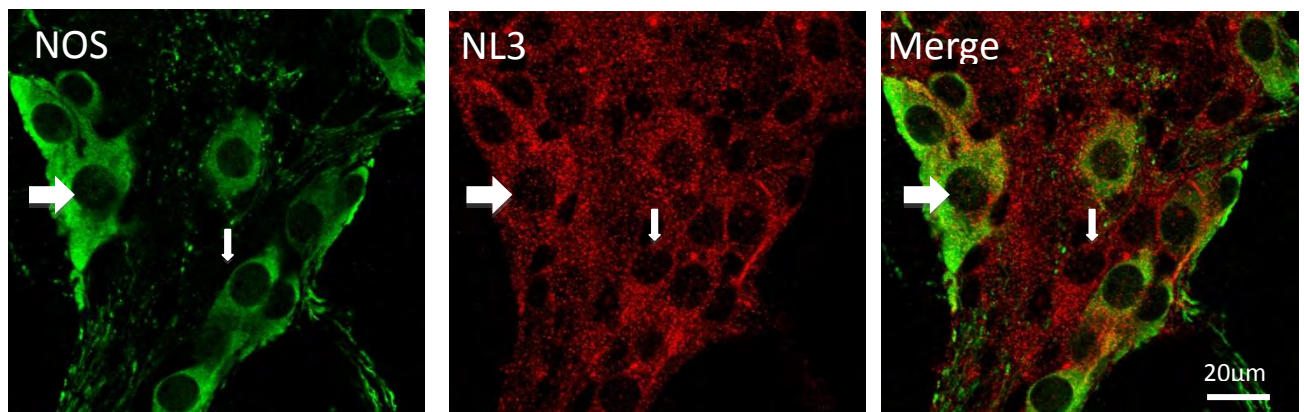
Figure 6: Localisation of nNOS and NL3 in the myenteric plexus of guinea pig. These images show clear neuronal localization of NL3 (red; 1:1000) with nNOS (green; 1:500) in neurons of the myenteric plexus. However, not all NL3 immuno positive neurons appear to express NOS (guinea pig myenteric plexus tissue).
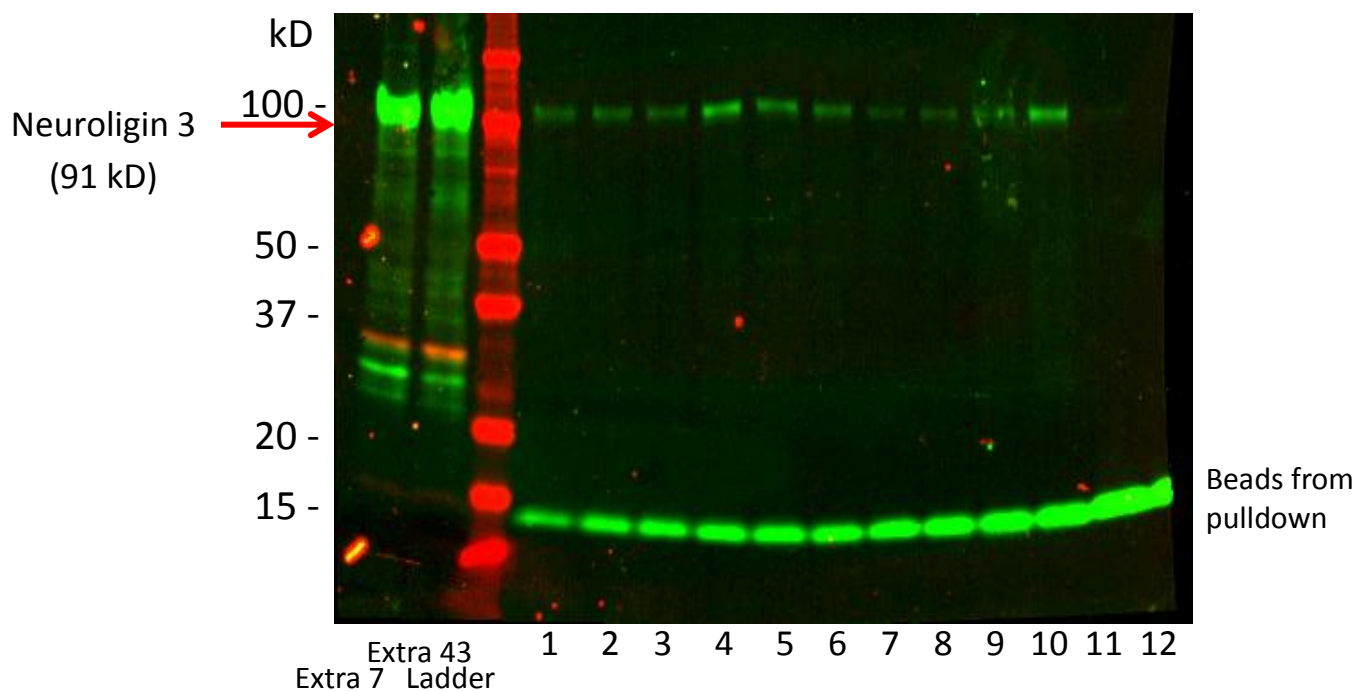

Figure 7: **Western blot showing specificity of the Neuroligin-3 antibody.** The Neuroligin-3 antibody (cat# 129 113 rabbit polyclonal, Synaptic Systems) labels a 91kD protein (predicted weight of Neuroligin-3) in mouse hippocampal tissue (lanes 1-12).

Developmental GI behavioural analyses

Based on scientific feedback that we should assess for behavioural GI phenotypes (it was suggested by Scientist reviewer A that the application would benefit from measuring actual *in vivo* GI function by using a routine charcoal transit assessment) we are the in the process of organising to conduct these experiments.

In accordance with this recommendation we plan to investigate whether behavioural GI parameters such as fecal pellet production (i.e. number and wet weight of pellets) and gastrointestinal transit is altered in NL3 mice compared to WT littermates. These experiments were included in a separate grant application to the NHMRC government funding body (however we were recently notified that this application was unsuccessful) and local animal ethics approval has been obtained. We plan to request that this work be added to the current project with no change to the budget.

Limitations

The localization of the NL3 protein in adult mouse ENS has yielded unexpected results and has delayed progress for the developmental component of the project. Due to our results showing presynaptic NL3 labelling we have moved to confirm the specificity of the commercial antibody using western blot and we are now optimizing immunohistochemistry protocols for use in developmental tissue based on the data obtained in adult mice.

Breeding mice has taken longer than envisaged, further limiting the number of experiments possible including those associated with SOW Task 4b; FACs analysis in developmental tissue.

Key Research Accomplishments

- Demonstration of expression of synaptic genes CNTNAP2, gephyrin and, importantly a binding partner of neuroligins, SHANK3 in mouse enteric tissue preparations

- Demonstrated upregulation of 3 genes implicated in ASD and epilepsy during development of the mouse ENS (CACNA1A, KCNQ3 and SCN1A)

- Neuronal proportions labelled for GABA, nNOS and the pan neuronal marker Hu are similar in WT and NL3 adult mouse colon suggesting that the NL3 R451C mutation impacts on synaptic function in the ENS without altering neuronal numbers during development.

- Finding NL3 in presynaptic terminals is unexpected and should lead to re-evaluation of its location at central synapses.

- These data also indicate that many neurons in mouse do not express neuroligin 3 in their cell bodies or dendrites. This contrasts with guinea pig and suggests that species differences may be very important.

Reportable Outcomes

Published manuscripts:

1. Hao MM, **Bornstein JC**, Vanden Berghe P, Lomax AE, **Young HM**, Foong JP. The emergence of neural activity and its role in the development of the enteric nervous system. Dev Biol. 2012 Dec 19. doi:pii: S0012-1606(12)00672-0. 10.1016/j.ydbio.2012.12.006. [Epub ahead of print]

2. Hao MM, **Bornstein JC**, **Young HM**. Development of myenteric cholinergic neurons in ChAT-Cre;R26R-YFP mice. J Comp Neurol. 2013 Oct 1;521(14):3358-70. doi: 10.1002/cne.23354. PMID:23649862

Conference Abstracts:

1. Digestive Diseases Week 2013. Symposium on "Regenerative Medicine and the Intestine". Orlando, May 2013 [invited presentation]

Invited Seminars

1. International Neurogastroenterology & Motility meeting, Bologna, Italy, Sept 2012; part funding.

2. Mead Johnson Pediatric Nutrition Institute sponsored conference on "Microbiome and the Brain - Co-development and Function", San Francisco, October 2012; full funding.

3. Enteric Nervous System II, Adelaide, Australia, February 2014; part funding

4. Hunter Cell Biology Meeting, Hunter Valley Australia, March 2014

Conclusion

Using qPCR, this work has identified that the expression of ion channel genes associated with ASD and epilepsy are upregulated during development. Specifically, the expression of the synaptic genes CACNA1C, KCNQ3 and SCN1A in the mouse gastrointestinal tract is increased during development compared to their respective expression levels at E11.5. Our data suggest that there may be a gastrointestinal phenotype involving ENS dysfunction in patients with both epilepsy and autism.

We have optimized labelling procedures and investigated the localization of the NL3 protein in mouse GI tissue. Due to unexpected results from these experiments in the mature ENS, we moved to confirm the specificity of the commercial antibody using western blot. As the mouse breeding has been unexpectedly slow, unavailability of tissue has prevented us proceeding with the FACs experiments. Accordingly, we will first complete the developmental localization of the protein.

We have recently been informed that our application for funding elsewhere (NHMRC) to investigate whether a GI phenotype can be detected *in vivo* during development was unsuccessful. However, we have local ethics approval to conduct these experiments and will submit an application to amend the current Statement of Work and ACURO approvals to include these behavioural experiments in the current project at no extra cost to the budget.

In conclusion, the immunohistochemical findings suggest that the neuroligin-3 protein may be present in presynaptic locations during development and that neuronal proportions are unchanged in the NL3 mice. These findings have major implications for understanding the role that NL3 plays during development. Data from the qPCR experiments suggest that it may be worthwhile assessing for GI phenotypes in patients with ASD and epilepsy expressing these and other relevant synaptic gene mutations. These novel findings support the idea that gene mutations that affect synaptic function in the central nervous system may alter neural communication in the ENS to influence GI motility.

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Appendices

Table 1. Summary of animals and antibodies used for 4a and 4c

Date	Mouse ID	Weight (g)	Sex	Genotype
13.08.13	210	26.72	Male	WT/O
14.08.13	170	24.16	Male	KI/O
27.08.13	245	31.53	Male	WT/O
28.08.13	223	29.17	Male	WT/O

Table 2. Antisera for Task 4c (immunohistochemistry in developing tissue).

Antiserum	Supplier	Cat. No.	Dilution
Rabbit Anti-Neuroligin 1	Santa Cruz	sc-50393	
Goat Anti-Neuroligin 2	Santa Cruz	sc-14089	
Rabbit Anti-Neuroligin 2	Synaptic Systems	129 203	
Rabbit Anti-Neuroligin 3	Synaptic Systems	129 113	1:1000 (Mouse) 1:500 (G.Pig)
Goat Anti-Neurexin 1	Santa Cruz	sc-14334	
Goat PSD-95	Santa Cruz	sc-6926	
Neuroligin 2 Control Peptide	Synaptic Systems	129 2P	
Neuroligin 3 Control Peptide	Synaptic Systems	129 3P	1:500 (G.Pig)
Sheep Anti NOS	Jackson West Grove USA		1:1000
Donkey anti Sheep Alexa 488 (green)	Life Technologies AustraliaInvitrogen		1:400
Donkey anti Rabbit 594 (green)	Life Technologies AustraliaInvitrogen		1:400

Biographical Sketch

Provide the following information for each individual included in the Research & Related Senior/Key Person Profile (Expanded) Form.			
NAME: HEATHER M. YOUNG		POSITION TITLE: PROFESSOR AND NHMRC SENIOR RESEARCH FELLOW	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
University of Melbourne, Australia	BSc (Hons)	1979	Zoology
University of Melbourne, Australia	PhD	1989	Neuroscience

Positions and Employment

1989-1990	Post-doctoral fellow, Vision, Touch and Hearing Research Centre, University of Queensland, Australia
1991-1993	Post-doctoral fellow, Department of Physiology, University of Melbourne, Australia
1994-1996	NHMRC RD Wright Research Fellow, Department of Anatomy & Cell Biology, University of Melbourne, Australia
1997-2000	NHMRC Research Fellow, Department of Anatomy & Cell Biology, University of Melbourne, Australia
2001-present	NHMRC Senior Research Fellow, Department of Anatomy & Cell Biology, University of Melbourne, Australia

Honours and experience

• Recipient of a "Janssen Award for Basic or Clinical Research in Digestive Sciences" in 2002. The awards are "dedicated to supporting excellence in gastroenterology".

• Associate Editor: *Autonomic Neuroscience;* Editorial Board member: *Histology & Histopathology.*

Publications in past 3 years (2010 onwards):

1. HOTTA, R., ANDERSON, R.B., KOBAYASHI, K., NEWGREEN, D.F. and **YOUNG, H.M.** (2010) Effects of age, presence of neurons and lack of endothelin-3 on the ability of enteric neural crest-derived cells to colonize explants of recipient gut: Implications for cell-based therapies. *Neurogastro Motil* **22**:331-e86

2. HAO, M.M., MOORE, R.E., ROBERTS, R.R., NGUYEN, T., FURNESS, J.B. ANDERSON, R.B., and **YOUNG, H.M.** (2010). The role of neural activity in the migration and differentiation of enteric neuron precursors. *Neurogastro Motil* 22:e127-37.

3. ROBERTS, R.R., ELLIS, M., GWYNNE, R.M., BERGNER, A.J., LEWIS, M., BECKETT, E.A., BORNSTEIN, J.C. and **YOUNG, H.M.** (2010). The first intestinal motility patterns in fetal mice are not mediated by neurons or interstitial cells of Cajal. *J Physiol.* 588:1153-69.

4. **YOUNG, H.M.,** CANE, K.N. and ANDERSON, C.R. (2011) Development of the autonomic nervous system: A comparative view. *Autonom Neurosci* 165:10-27.

5. McKEOWN, S.J., ANDERSON, C.R. and **YOUNG, H.M.** (2011). Highlights in basic autonomic neuroscience: Development of autonomic neurons – from the neural crest to circuit formation. *Autonom Neurosci* 165:149-152.

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myenteric neuron subtypes in the small intestine of the mouse. *J Comp Neurol* (in press, accepted 27.06.13).

Impact of research, contribution to training:

• h index = 43; Fifteen papers with >100 citations. Total of >5,200 citations. Average citations per publication: 43

• Career total of 113 peer-reviewed publications.

• Supervisor of nine post-graduate students who have completed PhDs.

Invited conference presentations in past 3 years:

1. *2010 Joint International Meeting in Neurogastroenterology and Motility;* Aug 2010, Boston, USA (partial funding)

2. *OzBio2010 (12th International Union of Biochemistry & Molecular Biology);* Sept 2010, Melbourne, Australia

3. *2012 Australian Neuroscience Society (ANS) plenary lecture*, Gold Coast, Australia. Feb 2012 (full funding)

4. *Third international symposium on Development of the Enteric Nervous System;* March 2012, Hong Kong (full funding)

5. *Digestive Diseases Week 2012*. Symposium on "Translational Medicine". San Diego, May 2012.

6. *International Neurogastroenterology & Motility meeting*, Bologna, Italy, Sept 2012; part funding.

7. *Mead Johnson Pediatric Nutrition Institute sponsored conference* on "Microbiome and the Brain - Co-development and Function", San Francisco, October 2012; full funding.

8. *Digestive Diseases Week 2013*. Symposium on "Regenerative Medicine and the Intestine". Orlando, May 2013.